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Reply to 'The interface of nanoparticles with proliferating mammalian cells'

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The work of Panet *et al.*[1] is a welcome addition to earlier reports on nanoparticle uptake in proliferating cells[2,3], and provides us the opportunity to further reinforce and clarify the main points of our paper[2] on the coupling of cell cycle progression and nanoparticle accumulation.

Firstly we are struck by the fact that many of the experimental results and trends Panet *et al.* report are consistent with our observations, despite the very considerable differences between systems and experimental approaches. It is indeed pleasing that, for instance, average uptake rates for different cell cycle phases – if properly evaluated in the linear regime – are very similar (Fig. 1a-b). Obviously these refer to average uptake rates, while there is a distribution around them on a cell-by-cell basis (potentially including due to cell size) as explained in our original paper. Given the great differences in cell type and approach used, the level of agreement is testament to the possibility to make useful measurements in this field.

We appreciate the technical approach the authors have taken in their paper, and while this is not the appropriate format for a detailed discussion of many technical details, we note a few arenas of interest. Possibly careful consideration might need to be given to particle effects on cellular side-scattering. This is particularly true for the highly convoluted (sub-micron scale) surface of cells, and to us the hypothesized relationship of this measurement to (appropriate) 'cell surface areas' is not obvious. Thus, since nanoparticles interact with the cell surface on that mesoscopic scale, the degree to which a fixed angle scattered light detector on a flow cytometer would capture these effects and accurately report an appropriately meaningful cell area and volume seems a question of some subtlety. Nowadays we are also aware that such cellular access is receptor driven, and another key issue that might be considered is whether the densities of receptors (*via* which particles enter) are fixed for all individual cells in all states and the degree to which this is required to establish useful measures of 'area' to nanoparticle access to receptors.

Indeed, the capacity of conventional live cell light microscopy to resolve where nanoparticles are, whether on the cell surface (where they spend much of their time), cell culture plate, or within the cell (especially given the convoluted cell surface) has always been doubted. We note very carefully that nanoparticles may be associated to the surface in complex manners, or be involved in other (transient) complex associations that are difficult to resolve, all of them for much longer times than usual for molecules. The difficulty of separating surface-associated particles (and whether external in the membrane folds or within the cell, and if so how far inside) will be recognized as a long-discussed challenge to bionanoscience investigations, in which the details and approach to 'washing' and many other details will be crucial. Many systematic electron microscopy studies of the convoluted nature of the nanoparticle-cell interface are sufficient warning against simple interpretations. Possibly, in that context also, one should be cautious to rely only on optical microscopy until all of these questions are seen to be resolved. One should most certainly not see these remarks as necessarily critical of the work of Panet *et al*. On the contrary, typical bionanoscience and many cellular studies are undertaken using quite different (and much more laborious) tools than those used by the authors, and the convenience of their approach will be welcomed. Still, since none of these issues is addressed by the authors, a more detailed understanding of the assumptions (and any limitations) inherent in these more convenient approaches would be a most welcome addition to the field.

There are other more practical issues also. We observe that the results of Panet *et al.* describing the number of nanoparticles per cell as a function of cell size (Fig. 1c-f, h and k; Fig. S5-6, S8-9, S13) have significant intercepts. Given that Panet *et al.* concludes that the nanoparticle concentration is constant, we would have presumed they would pass through the origin (assuming that auto-fluorescence vanishes with vanishing cell volume). We are thus unsure of what they actually base their main conclusion on.

Despite points of agreement, it also must be said that we expect some different outcomes than those suggested by Panet *et al*. For example, in line with our original paper we expect the effects of variation of cell surface area on uptake during the cell cycle to exert only a minor role for the effects we report. To illustrate this simply, consider two cells at either extreme of the cell cycle, a cell that has just been born and a cell that is just about to divide. Their volumes typically differ on average by a factor of roughly 2. If the uptake rate into cells is proportional to cell area, as suggested by Panet *et al.* (their Fig. 1i), then this implies only a factor of $2^{2/3} \approx 1.6$ of difference in uptake rates (Fig. 1c) – even at the extremes. A more complete analysis must take into account the simultaneous evolution of the cell population during nanoparticle exposure, as done in our previously published model.[2] Extending this model to include cell surface area changes (see Supplementary Information) allows us to compare uptake kinetics obtained by including or neglecting a surface area dependence of the nanoparticle uptake rate (Fig. 1d). Consistent with our original report, the results clearly show that differences in accumulation curves due to surface area changes are barely measurable within the errors of the methods used. This illustrates the point that, even when nanoparticle entry is made dependent on cell surface area (captured for example in a short term accumulation experiment), the time evolution in which different cells divide, and then continue to progress through their cell cycle while taking up particles, leads the results we report to be dominated by cell cycle progression, not by the cell area dependent rates. We stress that, of course, area changes *do* occur during the cell cycle, and by necessity they will introduce a correlation between nanoparticle load and cell 'volume', a significant point to which we return below. However, that correlation is there whether uptake rates are made dependent on cell surface area or not (Supplementary Fig. S4).

Panet *et al.* correctly highlight some semantic issues that deserve consideration. For instance, we used the phrase 'nanoparticle intracellular concentration' to mean number of nanoparticles *per cell*. Overall cell volume is, we feel, not the appropriate volume to discuss in the context of 'concentration' (since the nanoparticles are confined in small organelles where the effective concentration is very large). More importantly, in distinction to the case of small molecules, cellular 'concentration' does not couple to driving force (such as the chemical potential) in the cell energy-dependent distribution of nanoparticles. Cellular concentration thus defined is therefore of uncertain value as a concept. We are also unsure of the real meaning of the term 'cell size' used by Panet *et al.* unless it is connected to surface areas and volumes, an issue that has been discussed above. Equally, our original terminology is now quite outdated; nowadays knowledge has moved on and, as indicated above, we tend to think about microscopic processes, receptor densities, and other molecularly based parameters, rather than such macroscopic concepts, and we feel one may expect these semantic issues to evolve away naturally.

Returning to the underlying science, we stress that the central point of our original paper was to emphasize that nanoparticle accumulation is fundamentally coupled to cell cycle progression, beyond minor effects due to changing cell surface area. On this issue we appear to arrive at different conclusions compared to Panet *et al.* The reasons are not entirely clear. Presuming there is universal agreement on the key experimental results, irreversible accumulation of nanoparticles (the limited export of these particles; their Supplementary Fig. S15) and the sharing of those accumulated nanoparticles between daughter cells upon division (their Supplementary Fig. S14) then we argue that a coupling between cell cycle progression and nanoparticle accumulation is simply inevitable and leads to the results we reported in our original paper.

It is perhaps easier to understand this difference not under conditions of continuous exposure (Fig. 1d), where simultaneous uptake and cell cycle progression complicate interpretation, but rather after a pulsed exposure (Fig. 1e).[2,5] Thus, consider two cell populations, one exposed to nanoparticles that enter (irreversibly) by energy-dependent processes, and one exposed to conventional molecules that partition into and out of cells by

simple diffusion. After exposure, in both cases cells will contain, respectively, nanoparticles and molecules. However, upon removing the extracellular source, the difference between nanoparticles and molecules is profound. The molecules flow out rapidly (Fig. 1e; circles)[5] under diffusive control to re-adjust the concentration gradient, and the cell cycle plays no real role (except as a minor modulator of fluxes *via* cell area). On the contrary, the reduction of nanoparticle amounts in cells occurs solely by cell division (Fig. 1e; squares)[2] and is directly coupled – indeed slaved – to the time-scale of the cell cycle.

We note carefully the risk of conceptual confusion in these discussions. Thus, any parameter of cell structure or ultrastructure that contributes to nanoparticle accumulation, and which is also slaved to cell cycle (including, as noted above, cell volume) could *appear* to generate a correlation between that parameter and nanoparticle accumulation (*cf.* Supplementary Fig. S4). This is an elementary point, but worth reflecting on carefully. As a simple illustration, a dividing cell will split its contents – the (irreversibly) accumulated nanoparticle load and volume – between daughter cells, leading to a strong correlation between volume and nanoparticle content. It would be usual, though, to consider this correlation as being derived fundamentally from the progression of the cell cycle, rather than between volume and nanoparticle load. Additionally, there may (though we have no reason to suppose there is here) even be true intrinsic contributions from volume (distinct from and independent of the cell cycle). That is, quite simply cells with different volumes possessing different properties (for example variable import machinery, receptor densities *etc.*) accumulate particles differently (apart from the surface area effect). We note carefully that for this particular example, cell cycle progression driven volume correlations will combine with such variations in cell volumes, acting in the same direction (that is, giving the same ranking of accumulation). It will be very challenging to separate the contributions, and attributing them to volume dependent rates, as the authors seek to do. Our paper was quite clear on this point. While we do not exclude such contributions, we found (and find) no reason to invoke them, as the (certain) behavior of cell cycle progression is entirely sufficient to fully explain the observations. Should further satisfactory evidence emerge to suggest these 'co-operating effects' there could surely be no objection to such a report. However, one should be aware that there are many dozens of other such effects, ranging from receptor numbers and status to detailed microstructure of the cellular interface, that will also contribute.

We should also note carefully that apparent correlations between volume and accumulated particles may (as we consider in this case) have no deeper scientific significance. The inverse logic, that reversible partitioning and volume-compositional correlations are tied together is reasonable, whereas irreversible partitioning and cell cycle driven accumulation is entirely consistent to any volume correlation (including a linear one). Discussions in this direction of causation risk returning to the arena of semantics with multiple confusions if incorrectly posed. That is why it so important to return once more to the core science, and explore the question on the basis also of pulsed exposure experiments (such as Fig. 1e; squares)[5] rather than (near) steady-state correlations of cell parameters. The result is conclusive, in our view: nanoparticle accumulation is under control of the cell cycle.

While the issues raised by Panet *et al.* have been addressed in the intervening years from our original publication, some new questions, not discussed by either of our publications, have arisen and we have been studying them for several years. For instance, it is long known that smaller subpopulations of internalized nanoparticles adopt non-endo-lysosomal pathways[6], some transiting on cell export and recycling pathways. Such transient subpopulations would be lost to the cells on removal of the particle source, and their measurement will depend on washing and preparation steps and many other technical details of the experiment. Their role is a topic of some interest. Nevertheless, the experiments in our original paper were intended to abstract and highlight the key conclusion that particle accumulation is coupled to the cell cycle. They were set up (using specifically selected approaches) to eliminate such potentially ill-defined and transient nanoparticle

subpopulations and demonstrate that the major well-defined population of internalized nanoparticles, once accurately identified, is irreversibly accumulated in lysosomes, and dilution in cells is slaved to cell cycle.

In summary, while many results in the two papers are in striking agreement (given the great differences in approach) there are still some differences to be resolved. We do appreciate and respect the fact that different perspectives and different communities of scientists will consider different issues of importance, and there are indeed countless parameters of interest for different scientists in this arena. However, for the reasons above, we do not expect explicit inclusion of cell surface area changes during cell cycle to substantively affect our reported results, nor any substantive scientific conclusions (of the type we considered). Rather, we believe those effects can be subordinated, as with many other genuine cellular effects, in capturing the major outcomes of cellular accumulation. This is fortunate since it is a highly challenging task to define and reliably measure real (and appropriate) highly folded surface areas through which nano-sized objects navigate. Secondly, significantly, we consider that the population of nanoparticles that accumulates in cells is most certainly coupled to the cell cycle, and is captured purposefully and accurately by the methods described in our original paper, and leads to the cell cycle phase ranking reported there. Other experimental approaches allowing the capture of more complex or transient nanoparticle-cell associations could lead to a variety of different potential (possibly poorly-defined) volume correlations, depending on many detailed choices made in the measurement and analysis. While such investigations are to be welcomed, in our view, it is most important that reports on them do not suggest an outcome comparable to that seen for reversibly cell-associating small molecules where ‘equilibrium’ is expected, and consequently accumulation typically scales with volume. Such conclusions would miss the major scientific point that cell cycle and nanoparticle accumulation are intrinsically coupled, and qualitatively different from the molecular picture of cellular uptake.

This conceptual distinction is as important for us to stress now as it was in our original paper, because of its significant scientific and practical implications. Thus, for molecular drugs there is little point in trying to target the cell cycle, since when the drug source is removed accumulated molecules are rapidly re-partitioned, and the drug washed into the blood-stream. However, since intracellular nanoparticle accumulation is fundamentally coupled to cell cycle progression, targeting cycling cells is in principle possible and only demands design of nanoparticles that, for example, engage with cell cycle phase-specific surface markers. Upon removal of the source, those that thereby accumulate irreversibly will not repartition or ‘leak’ away rapidly. This fundamentally novel potential makes targeting of cycling cells (*e.g.*, in tumours) viable in an inherently new way.

We hope this has fully clarified certain key scientific issues in relation to the coupling of cell cycle progression and nanoparticle accumulation. We also expect further detail (reflected in molecular mechanisms) on the nature of the coupling of the cell cycle to nanoparticle uptake and accumulation to emerge in future. Let us also stress that many other aspects of this arena have important new science as yet unexplored or poorly understood, and are of considerable practical and fundamental interest.

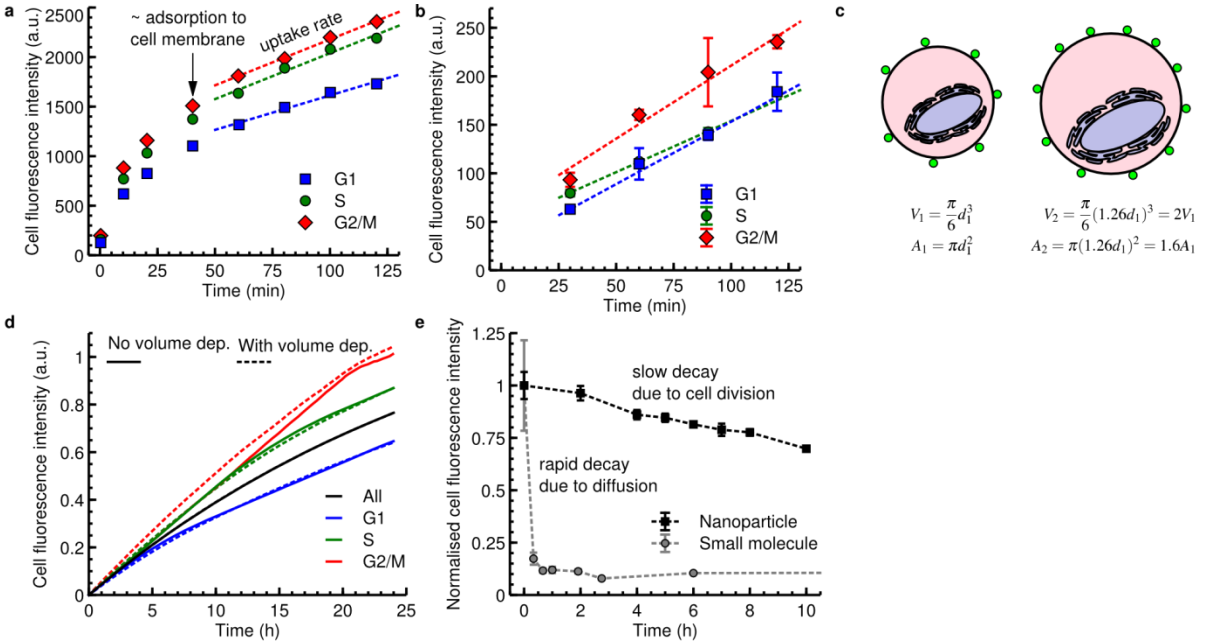

Figure 1. Nanoparticle accumulation in proliferating cells. a, Uptake rates in L1210 cells of different phases. (Symbols) Data from Panet *et al.* (their Fig. 1j). (Lines) Our fits to determine uptake rates, giving rates in proportion 1.0:1.3:1.3 for G1, S and G2/M cells, respectively. We stress the difference between the *amount* of nanoparticles associated with cells (cell fluorescence) and nanoparticle *uptake rate*. The uptake rate must be evaluated from the linear part of the curve; we used the data after 40 or 60 min, in both cases getting the same proportions. The ranking in cell fluorescence, on the other hand, must be interpreted with care. Certainly, a large part of the signal at these short exposure times may come from nanoparticles adsorbed to the outer cell membrane, as we have shown previously[4]. This is consistent with larger (G2/M) cells exhibiting a higher fluorescence than smaller (G1) cells. Additionally, there may be a range of other transient phenomena, currently not well understood. **b,** Data from A549 cells and fits reproduced from our previous work (our Fig. 4) giving rates in proportion 1.0:0.8:1.2. Both works agree that uptake rates are roughly the same in all cell cycle phases (panels a-b). **c,** Schematic illustrating the small effect of cell volume changes during cell cycle on nanoparticle uptake. Two spherical cells, one (left) with half the volume of the other (right). A difference of a factor of 2 in volume gives only a factor of 1.26 in diameter and a factor of 1.6 in area. For simplicity the cells have been drawn spherical, but this is not central to the argument. **d,** Nanoparticle accumulation as a function of time in simulated A549 cells, assuming nanoparticle uptake rate (solid lines) independent of cell area and phase or (dotted lines) proportional to cell area. Results are shown for cells that are at any given moment in a given cell cycle phase. The simulations were performed using parameters for A549 cells from our previous work (see Supplementary Information for details). The results show that the overall uptake kinetics for cells in different cell cycle phases are roughly the same, regardless of whether the uptake rate changes with cell area or not. **e,** Experimental data comparing how the accumulated amount of small molecules and nanoparticles change as a function of time after removal of the source. While small molecules diffuse out of the cells within less than 1 h, the (irreversibly) accumulated nanoparticle load does not exit and decreases slowly over several hours solely due to cell division. Data for small molecules reproduced from ref. 5 (Fig. 4B; YG) and for nanoparticles from ref. 2 (Fig 3C).

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